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Abstract: We hypothesize that REP cells are neuron-like setpoint providers and controllers, which integrate information about blood O2 concentration and local O2 consumption via tissue pO2, and combine these inputs with intrinsic negative feedback loops and perhaps tubular cross-talk, converging in Epo regulation.

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Source and microenvironmental regulation of erythropoietin in the kidney

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Purpose of review
Historically, the identity of O_2-sensing renal erythropoietin (Epo)-producing (REP) cells was a matter of debate. This review summarizes how recent breakthroughs in transgenic mouse and in-situ hybridization techniques have facilitated sensitive and specific detection of REP cells and accelerated advancements in the understanding of the regulation of renal Epo production in health and disease.

Recent findings
REP cells are a dynamically regulated unique subpopulation of tubulointerstitial cells with features of fibroblasts, pericytes and neurons. Under normal conditions, REP cells are located in the corticomedullary border region within a steep decrement in O_2 availability. During the progression of chronic kidney disease (CKD), REP cells cease Epo production, dedifferentiate and contribute to the progression of renal fibrosis. However, CKD patients with renal anaemia still respond with elevated Epo production following treatment with hypoxia-mimicking agents.

Summary
We hypothesize that REP cells are neuron-like setpoint providers and controllers, which integrate information about blood O_2 concentration and local O_2 consumption via tissue pO_2, and combine these inputs with intrinsic negative feedback loops and perhaps tubular cross-talk, converging in Epo regulation.

Keywords
chronic kidney disease, hypoxia-inducible factor, oxygen, prolyl hydroxylase inhibitor, renal anaemia

INTRODUCTION

Erythrocytes, the body's vehicle for O_2 transport, are continuously replaced by fresh cells. Each day, 20 ml of red blood cells (RBCs) are required to replace the approximately 120-day-old erythrocytes removed by the reticuloendothelial system [1,2]. In the adult, kidney-derived erythropoietin (Epo) is the essential regulator of RBC formation not only under physiological conditions but also during instances of increased blood loss or reduced O_2 saturation of haemoglobin, for example at high altitude. Epo signalling through its receptor promotes the survival, proliferation and differentiation of erythroblasts, and under chronic conditions can increase the rate of RBC production up to 7-fold within days [2]. Plasma Epo levels can in fact increase several hundred folds in response to hypoxia [3]. However, for the maintenance of a normal haematocrit and physiological O_2 homeostasis in the healthy individual, only low basal levels of plasma Epo are required, which is produced by a small number of renal Epo-producing (REP) cells. Today, the connection between tissue pO_2, Epo production and RBC mass is clearly defined [2].

Epo expression is regulated almost solely at the transcriptional level by the hypoxia-inducible factor (HIF) pathway. HIF-regulated gene expression is critical for the general response to hypoxia, and HIFs ensure cellular adaptation, which protects cells and tissues from reduced O_2 availability. O_2 is sensed via O_2-dependent protein hydroxylation by the prolyl-4-hydroxylase domain (PHD) enzymes 1–3. In the presence of O_2, these enzymes hydroxylate the HIF α subunits. Hydroxylated α subunits are degraded by the proteasome following von Hippel–Lindau (VHL)-mediated polyubiquitylation. In addition, the factor-inhibiting HIF (FIH) attenuates HIF transcriptional activity by asparagine hydroxylation under normoxic conditions [4]. In renal

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KEY POINTS

- Renal Epo-producing (REP) cells are a subpopulation of cortical tubulointerstitial cells, sense drops in tissue pO_2 and respond with increased Epo expression.
- Genetic tagging and novel in-situ hybridization techniques unequivocally demonstrated that REP cells share features of fibroblasts, pericytes and neurons.
- Mouse models targeting REP cells demonstrate their contribution to the progression of renal fibrosis in CKD.
- Pharmacological activation of the HIF-pathway reverses loss of Epo production in CKD and is an attractive therapeutic option for the treatment of renal anaemia.

tubular cells, the PHD/VHL pathway mainly regulates HIF-1α, which in turn controls O_2 consumption and energy metabolism. In cortical interstitial cells, following a decrease in arterial O_2 concentration, HIF-2α is stabilized and induces Epo gene expression [5]. Indeed, conditional knockout of HIF-2α in adult mice results in anemia, whereas loss of HIF-1α does not [6]. Among the many hundreds of HIF target genes, Epo is regulated with particular sensitivity. In hypoxia, HIF-2α regulates a rapid and robust Epo mRNA induction, which decreases despite ongoing hypoxia [7,8]. This is in contrast to the delayed and prolonged HIF-2α expression commonly observed in cancer cell lines [9], and suggests a unique regulation of HIF-2α in REP cells. PHDs are HIF targets themselves and are likely involved in the negative feedback on Epo mRNA expression as reported previously for other HIF target genes in cancer cells [10].

ERYTHROPOIETIN PRODUCTION: WHY THE ADULT KIDNEY?

In the developing embryo, the liver is responsible for the production of circulating Epo. Epo and EpoR knockout mice die in utero, highlighting the importance of Epo in normal development [11,12]. Postnatally, a switch in the site of Epo production occurs, with the kidney becoming the predominant organ for Epo production, accounting for approximately 90% of total Epo synthesis in the adult [2]. Under physiological conditions, the ratio between renal O_2 supply and consumption stays within a relatively narrow range. Unlike other organs, which experience enhanced tissue oxygenation upon increased blood flow, the kidney does not. The reason for this is the tight matching between glomerular filtration rate (GFR) and transport processes. Increasing renal blood flow (i.e. more O_2 supply) increases GFR and reabsorption (i.e. more O_2 demand). The resulting relatively stable oxygenation makes the kidney a superior organ for sensing decreases in tissue pO_2 caused by decreased blood O_2 concentration [4,13]. However, it should be noted that if the O_2-carrying capacity of the blood increases, it does result in improved oxygenation, even at constant renal blood flow. A recent study by Dimke *et al.* [14] highlighted the importance of appropriate kidney perfusion and hence, oxygenation in renal Epo expression using a mouse model lacking tubular VEGFA. These mice developed smaller kidneys with a decreased density of peritubular capillaries and were polycythaemic because of enhanced renal Epo production. Although other organs such as the liver, brain, testes, uterus and adrenal glands have also been reported to produce Epo in the adult, they contribute only to a minor degree to circulating Epo [15–18,19▪].

RENAL ERYTHROPOIETIN-PRODUCING CELLS: INSIGHTS FROM GENETICALLY MODIFIED MICE

The first undisputable evidence that REP cells are a population of interstitial cells found mainly in the cortex and the outer medulla came with the dawn of genetically modified mice. In 1993, Maxwell *et al.* [20] generated a mouse in which the Epo locus directed the expression of the marker gene SV40 T-antigen, which could be subsequently detected by immunohistochemical techniques. More recent developments, including the generation of fluorescent reporter mice using bacterial artificial chromosomes (BACs) of approximately 200 kb length as well as novel in-situ hybridization methods, have advanced the understanding of the REP cell population and further confirmed these initial in-vivo findings. Obara *et al.* [21] generated a transgenic mouse using a BAC clone in which the region spanning exon II to intron IV of the *Epo* gene was replaced with a green fluorescent protein (GFP) reporter gene. Consistent with the observations by Maxwell and colleagues, Obara *et al.* identified a GFP-positive sub-population of peritubular interstitial cells induced under anaemic conditions. Interestingly, these cells have dendrite-like processes and show features of neuronal cells, including the expression of MAP2 and NFL, as well as fibroblast markers CD73 and PDGFR-β [21,22]. Although this model was useful for the investigation of cells acutely producing Epo, the fate of these cells over time cannot be investigated, as the GFP expression is limited to the timeframe of active Epo transcription. In another approach to identify REPs, GFP cDNA was introduced into the endogenous Epo locus,

confirming the phenotype observed previously [23]. Finally, a recently developed improved in-situ hybridization technique revealed a fully consistent REP cell-staining pattern based on endogenous Epo mRNA [19▪,24▪].

Under genetically induced severe anaemic conditions, the majority of interstitial cortical fibroblasts produce Epo, as shown by Yamazaki *et al.* [25] using an Epo-Cre BAC transgene crossed to a tdTomato reporter mouse. However, at a given time point, only 10% of these cells are actively transcribing Epo. Similarly, whenever VHL is deleted in PDGFRβ-expressing interstitial cells, virtually all cells do express Epo [19▪]. Of note, genetic deletion of VHL from normally non-Epo producing cells also leads to Epo expression [26,27]. The physiological relevance of such genetic models, which artificially induce permanent and severe changes, has to be carefully considered as they fail to recapitulate the rather mild and transient physiological stimuli and the changing environment regulating the cellular hypoxic response.

Most recently, Epo-producing cells in the adult mouse kidney were found to be derived from FoxD1-expressing stromal cells. FoxD1 is expressed by the developing (but not the adult) kidney, and gives rise to cortical and medullary renal interstitial fibroblasts, pericytes, mesangial cells and vascular smooth muscle cells. *Epo* knockout in FoxD1-expressing cells had no impact on mouse development. However, these mice go on to develop hypoproliferative anaemia [24▪]. Furthermore, these mice showed no kidney Epo induction following exposure to 10% O_2 for 2 days or following treatment with the PHD inhibitor (PHI) GSK1002083A. The authors suggest that the small increase in serum Epo observed in PHI-treated mice is likely a result of liver-derived Epo. These data reveal that the relevant REP cell population is contained within the FoxD1-derived cell population. Interestingly, the FoxD1 population was found to be heterogeneous in that not all FoxD1-derived cells respond to Epo-inducing stimuli with Epo production. It was suggested that the different capacities for Epo production among the cells may be dependent on the PHD2 and PHD3 levels.

Together these studies unequivocally confirm the localization of REP cells in the deep juxtamedullay cortex under normal physiological conditions. This population expands to include more superficially located tubular interstitial cells under conditions of reduced tissue pO_2 (Fig. 1). However, REP cells fail to produce sufficient Epo to maintain a normal haematocrit in chronic kidney disease (CKD) patients.

RENAL ERYTHROPOIETIN-PRODUCING CELLS AND ERYTHROPOIETIN PRODUCTION IN CHRONIC KIDNEY DISEASE

Renal anaemia because of Epo insufficiency is a common complication of CKD, which increases in prevalence with the decline in estimated GFR

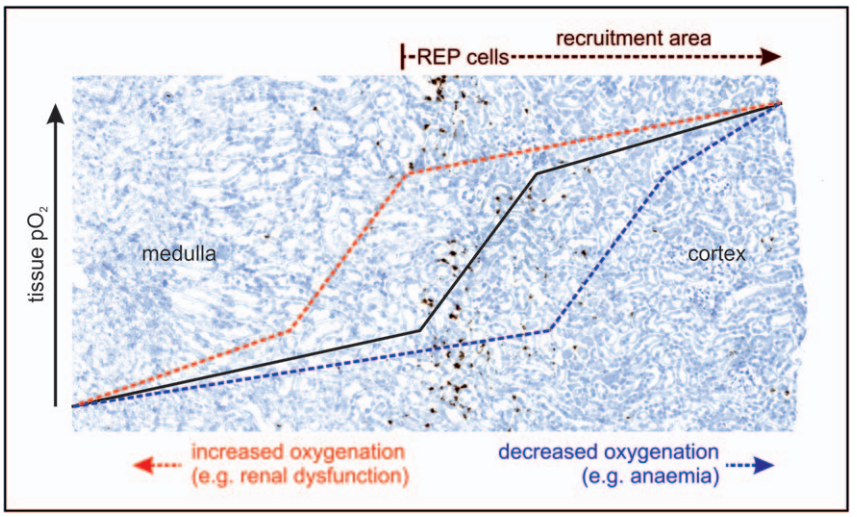

FIGURE 1. Schematic illustrating the location of renal erythropoietin-producing cells between the medullary and cortical areas. REP cells (brown; counterstained by hematoxylin, blue) were detected by Epo mRNA in-situ hybridization following exposure of a mouse to 0.1% carbon monoxide for 4 h, resulting in approximately 50% HbCO. Hypothetical tissue pO_2 profiles are indicated, including possible left and right shifts under conditions of increased or decreased, respectively, renal oxygenation. Whereas a right shift leads to the recruitment of additional REP cells, a left shift leads to the loss of Epo production as the medulla does not contain REP cells. Epo, erythropoietin; REP, renal erythropoietin-producing.

(eGFR), with over 50% of stage-5 CKD patients being anaemic [28]. Anaemic patients with normal renal function have 10–100-fold higher plasma Epo concentrations compared with anaemic CKD patients [29,30], supporting the idea that kidney Epo is the predominant source of circulating Epo in the adult human.

There are two apparently contradicting hypotheses explaining Epo dysregulation in CKD. On one hand, dedifferentiation of REP cells might cause a transient or permanent loss of Epo production despite lowered tissue oxygenation (e.g. because of vascular injury and/or fibrotic tissue remodelling). As discussed further in the following, perivascular cells, including REP cells are thought to be the precursors of myofibroblasts in CKD [31,32,33▪,34]. On the other hand, the reduced energy requirements of dysfunctional nephrons cause relative hyperoxia, HIFα degradation and ceased Epo production. In this case, we assume that REP cells do not lose their ability to produce Epo *per se*, but exist in a microenvironment which no longer supports Epo production. Interestingly, patients with kidney failure living at high-altitude require lower doses of recombinant Epo [35] and dialysis patients respond with increased Epo production to PHI treatment [36], demonstrating that the diseased kidney maintains the potential to express Epo. In addition to dedifferentiation and hyperoxia also other currently unknown tubular signals and reversible dedifferentiation might be involved in dysregulated Epo expression by REP cells. Recently, a role for tubular HIF-1α in Epo insufficiency has been proposed, suggesting a more complex crosstalk in the tubulointerstitial microenvironment [37].

Similar to these seemingly contradictory hypotheses for Epo insufficiency, the role of HIF in CKD and the progression of renal fibrosis is equally debated [38]. Evidence supports both protective as well as deleterious mechanisms of HIF, despite the fact that HIF clearly fails to induce renal Epo under these conditions. The conflicting findings likely reflect a very tightly regulated cell and context-specific function of HIF, which makes it difficult to define its exact role in CKD. Evidence to date is based on single snap shots of the complex pathway of disease progression and likely fails to reflect the dynamic HIF regulation.

In a recent study, a group of CKD patients with moderate anaemia was found to have higher Epo levels than healthy controls. de Seigneux *et al.* [39] demonstrated that Epo derived from these patients exhibits a different glycosylation pattern compared with Epo derived from healthy controls, but similar to the pattern of Epo derived from umbilical cord blood, which is considered hepatic in origin. These data suggest that although compensating for reduced circulating Epo, liver-derived Epo fails to correct renal anaemia.

In mouse models of renal injury, REP cells have been shown to transform into extracellular matrix producing myofibroblasts and contribute to the progression of renal fibrosis. Genetically tagged REP cells have been found to express the myofibroblast marker α-SMA in fibrotic but not in healthy kidneys [22,32,40]. The FoxD1 population of cells, reported to include REP cells [24▪], has previously been shown to be the predominant source of α-SMA-positive myofibroblasts, causing renal fibrosis [31,41]. Importantly, these myofibroblasts maintain the plasticity to resume Epo production, following removal of the injury-triggering stimulus or suppression of inflammation [32]. Also PHD2 knockout rescued Epo production but had no protective effect against the progression of renal fibrosis [33▪]. Interestingly, under disease conditions, the long REP cell processes, which are normally tightly wrapped around the capillaries, detach from the vessels and associate with adjacent tubules [33▪].

The role of O_2 (re-)distribution, however, if any, in the REP-myofibroblast differentiation balance is currently unclear because of technical limitations in determining the tissue pO_2 profiles at high resolution [42].

TARGETING THE OXYGEN-SENSING PATHWAY FOR THERAPY

Current therapy for patients with renal anaemia requires the chronic subcutaneous injection of recombinant human Epo, which is associated with increased blood pressure and cardiovascular risk [43]. Not surprisingly, the O_2-sensing pathway is an attractive target for the treatment of renal anaemia. In the last decade, small molecule HIF-stabilizing PHIs have been developed, aiming for an oral therapy enhancing endogenous Epo production. Several PHIs are currently in phase III clinical trials [44,45]. In addition to Epo stimulation, these compounds also have beneficial effects on iron metabolism. In contrast to sub-cutaneous recombinant Epo application, which results in peaks in plasma Epo concentration and may underlie adverse outcomes [46], PHI treatment results in smaller fluctuations in plasma Epo levels [47–49]. Long-term treatment of mice and rats with the PHI FG-4592/roxadustat has no effect on the development of neoplastic lesions [50] and does not promote tumour initiation, progression or metastasis in a VEGF-sensitive model of spontaneous breast cancer [51]. However, a cautious outlook remains until the potential nonerythropoietic side effects because of activation of the

HIF or other pathways [52] have been thoroughly addressed.

CONCLUSION

Historic disputes over the cell type responsible for the synthesis of kidney-derived Epo have truly been put to rest in the age of genetically modified mice and reliable in-situ hybridization techniques, which reproducibly identify a subpopulation of tubulointerstitial cells as REPs (Fig. 1). However, identifying unique characteristics of these cells, which separates them from the heterogeneous interstitial cell population has proven an exceptional challenge. Efforts are required to understand the microenvironmental changes that contribute to Epo suppression in CKD and likely involve the dysregulation of the HIF pathway in REP cells as well as their de-differentiation. Emerging therapies targeting the O_2-sensing pathway offer hope of an oral therapy for anaemic CKD patients and may be harnessed for therapeutic effects beyond the stimulation of renal Epo expression, including anti-inflammatory and antifibrotic effects.

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Conflicts of interest

There are no conflicts of interests.

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